Probe Report

Title: Discovery of a Highly Selective *in vitro* and *in vivo* M_4 Positive Allosteric Modulator (PAM) Series with Greatly Improved Human Receptor Activity

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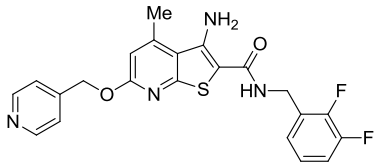
PubChem Summary Bioassay Identifier (AID): 2616

Abstract

This probe (ML173, CID 45142486) can be used both *in vitro* and *in vivo* to study the role of selective M_4 receptor activation. This probe possesses excellent selectivity versus M_1, M_2, M_3 and M_5, as well as a large panel of G-protein-coupled receptors (GPCRs), ion channels, and transporters. Moreover, this probe displays an order of magnitude greater potency at the human M_4 receptor versus the rat M_4 receptor (human M_4 EC_{50} = 95 nM) surpassing our initial M_4 positive allosteric modulator (PAM) probe, and now affects a large fold shift at both the human (60x) and rat (44x) receptors. While close analogs are active in rodent preclinical antipsychotic behavioral models, the greatly improved human potency may warrant testing in antipsychotic behavioral models with non-human primates. ML173 possess improved metabolic stability over the earlier M_4 PAM probe (CID 864492) making it a potentially better *in vivo* tool.

Probe Structure & Characteristics

3-amino-*N*-(2,3-difluorobenzyl)-4-methyl-6-(pyridin-4-ylmethoxy)thieno[2,3-b]pyridine-2-carboxamide, MW = 440.5, logP = 3.1, tPSA = 90.1 Å^2



ML173

CID/ML#	Target Name	IC_{50}/EC_{50} (nM) [SID, AID]	Anti-target Name(s)	IC_{50}/EC_{50} (μM) [SID, AID]	Fold Selective	Secondary Assay(s) Name: IC_{50}/EC_{50} (nM) [SID, AID]§
CID 45142486 / ML173	rM_4 hM_4	2440 95 [SID 92392533, AID 2616]	M_1, M_2, M_3, M_5	> 30 μM [SID 92392533, AID 1932, AID 1930, AID 1929, AID 1928]	> 100	ACh Fold-Shift (44-fold) [SID 92392533, AID 449769]

Recommendations for Scientific Use of the Probe

This probe (ML173, CID 45142486) can be used both *in vitro* and *in vivo* to study the role of selective M_4 receptor activation. This probe possesses excellent selectivity versus M_1, M_2, M_3 and M_5, as well as a large panel of GPCRs, ion channels and transporters. Moreover, this probe displays an order of magnitude greater potency at the human M_4 receptor versus the rat M_4 receptor (human M_4 EC_{50} = 95 nM) surpassing our initial M_4 PAM probe, and now affects a large fold shift at both the human (60x) and rat (44x) receptors. While close analogs are active in rodent preclinical antipsychotic behavioral models, the greatly improved human potency may warrant testing in antipsychotic behavioral models with non-human primates. ML173 possess improved metabolic stability over the earlier M_4 PAM probe (CID 864492) making it a potentially better *in vivo* tool.

1 Introduction

Specific AIM: To identify small molecule positive allosteric modulators and/or allosteric agonists of the M_4 muscarinic acetylcholine receptor that are cell permeable, possess low- to submicromolar potency and show greater than 10-fold selectivity over the other mAChRs (M_1, M_2, M_3 and M_5) employing a functional HTS approach.

Significance: To date, five muscarinic acetylcholine receptor (mAChR) subtypes have been identified (M_1–M_5) and play important roles in mediating the actions of acetylcholine (ACh) in the peripheral and central nervous systems.[1] Of these, M_1 and M_4 are the most heavily expressed in the CNS and represent attractive therapeutic targets for cognition, Alzheimer's disease, and schizophrenia.[2-4] In contrast, the adverse effects of cholinergic agents are thought to be primarily due to activation of peripheral M_2 and M_3 mAChRs.[5,6] Due to the high sequence homology and conservation of the orthosteric ACh binding site among the mAChR subtypes, development of chemical agents that are selective for a single subtype has been largely unsuccessful, and in the absence of highly selective activators of M_4, it has been impossible to test the role of selective M_4 activation. Clinical trials with xanomeline, a M_1/M_4-preferring orthosteric agonist, demonstrated efficacy as both a cognition-enhancing agent and an antipsychotic agent.[7-9] In follow-up studies in rats, xanomeline displayed an antipsychotic-like profile comparable to clozapine.[10] However, a long standing question concerns whether or not the antipsychotic efficacy or antipsychotic-like activity in animal models is mediated by activation of M_1, M_4, or a combination of both receptors. Data from mAChR knockout mice led to the suggestion that a selective M_1 agonist would be beneficial for cognition, whereas an M_4 agonist would provide antipsychotic activity for the treatment of schizophrenia.[5,6,11] This proposal is further supported by recent studies demonstrating that M_4 receptors modulate the dynamics of cholinergic and dopaminergic neuro-transmission and that loss of M_4 function results in a state of dopamine hyperfunction.[12] These data, coupled with findings that schizophrenic patients have altered hippocampal M_4 but not M_1 receptor expression,[13] suggest that selective activators of M_4 may provide a novel treatment strategy for schizophrenia patients. However, multiple studies suggest that M_1 may also play an important role in the antipsychotic effects of mAChR agonists and that the relative contributions of M_1 and M_4 to the antipsychotic efficacy of xanomeline or antipsychotic-like effects of this compound in animal models are not known. Unfortunately, highly selective centrally penetrant activators of either M_1 or M_4 have not been available, making it impossible to determine the *in vivo* effects of selective activation of these receptors. Only recently did we develop a highly selective M_1 allosteric agonist probe (CID 25010775) and two highly selective M1 PAM probes (CID 44251556 and CID 44475955) along with our initial M_4 PAM probe (CID 864492) to study the role of selective M_1 and/or M_4 activation *in vitro* and *in vivo*. Additionally, selective M_5 activation can now be studied using our most recent, highly selective M_5 PAM probe (CID 42633508).

Rationale: In recent years, major advances have been made in the discovery of highly selective agonists of other GPCRs that act at an allosteric site rather than the orthosteric site,[14] as well as positive allosteric modulators (PAMs).[15,16] By screening for compounds that act at an allosteric site on the receptor, it is anticipated that compounds that can selectively activate the M_4 receptor versus the other mAChR subtypes may be identified. In conjunction with our MLPCN M_1 allosteric agonist probe (CID 25010775), development of a potent and selective M_4 allosteric agonist or PAM will enable the biomedical community to dissect the pharmacology of xanomeline and determine the pharmacology and therapeutic potential of selective M_1 and M_4 activation.

2 Materials and Methods

2.1 Assays

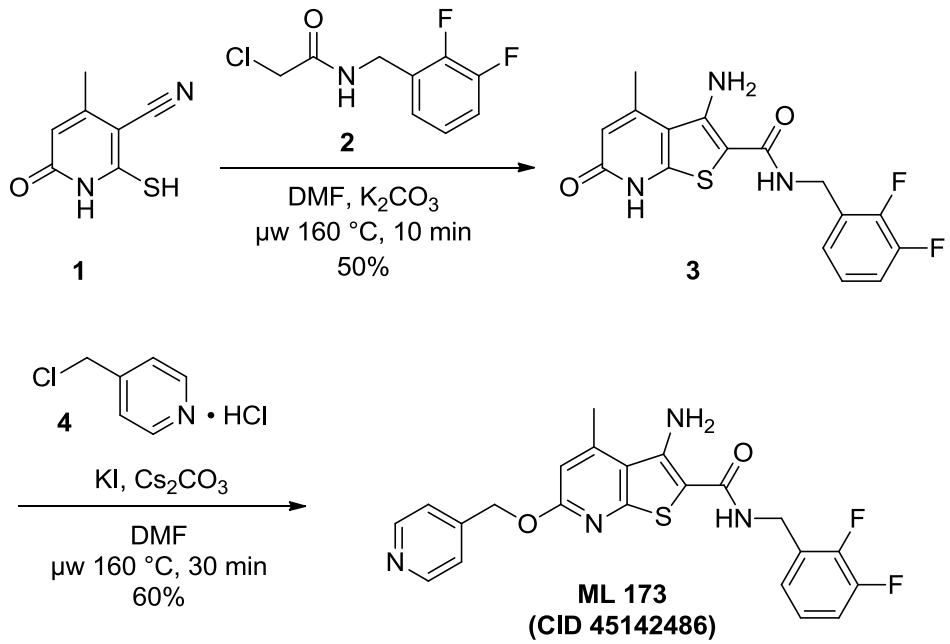
PubChem Primary Assay Description: Chinese hamster ovary (CHO K1) cells stably expressing rat (r)M_1 were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and cultured according to their recommendations. CHO cells stably expressing human (h) M_2, hM_3, and hM_5 were generously provided by A. Levey (Emory University, Atlanta, GA); rM_4 cDNA provided by T. I. Bonner (National Institutes of Health, Bethesda, MD) was used to stably transfect CHO-K1 cells purchased from the ATCC using Lipofectamine 2000. To make stable hM_2 and rM_4 cell lines for use in calcium mobilization assays, cell lines were cotransfected with a chimeric G protein (G_{qi5}) using Lipofectamine 2000. rM_2, hM_3, and hM_5 cells were grown in Ham's F-12 medium containing 10% heat-inactivated fetal bovine serum, 2 mM GlutaMax I, 20 mM HEPES, and 50 µg/mL G418 sulfate. hM_2-G_{qi5} cells were grown in the same medium supplemented with 500 µg/mL hygromycin B. Stable rM_4 cells were grown in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum, 2 mM GlutaMax I, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 20 mM HEPES, and 400 µg/mL G418 sulfate; rM_4-G_{qi5} cells were grown in the same medium supplemented with 500 µg/mL hygromycin B. CHO cells stably expressing rM_1, hM_3, or hM_5 were plated at a seeding density of 50,000 cells/100 µL/well. CHO cells stably coexpressing hM_2/G_{qi5} and rM_4/G_{qi5} were plated at a seeding density of 60,000 cells/100 µL/well. For calcium mobilization, cells were incubated in antibiotic-free medium overnight at 37 °C/5% CO_2 and assayed the next day.

Calcium Mobilization Assay: Cells were loaded with calcium indicator dye [2 µM Fluo-4 acetoxymethyl ester (50 µL/well) prepared as a stock in DMSO and mixed in a 1:1 ratio with 10% Pluronic acid F-127 in assay buffer (1xHanks' balanced salt solution supplemented with 20 mM HEPES and 2.5 mM probenecid, pH 7.4)] for 45 min at 37 °C. Dye was removed and replaced with the appropriate volume of assay buffer. All compounds were serially diluted in assay buffer for a final 2x stock in 0.6% DMSO. This stock was then added to the assay plate for a final DMSO concentration of 0.3%. Acetylcholine (EC_{20} concentration or full dose-response curve) was prepared at a 10x stock solution in assay buffer before addition to assay plates. Calcium mobilization was measured at 25 °C using a FLEXstation II (Molecular Devices, Sunnyvale, CA). Cells were preincubated with test compound (or vehicle) for 1.5 min before the addition of the agonist, acetylcholine. Cells were then stimulated for 50 s with a submaximal concentration (EC_{20}) or a full dose-response curve of acetylcholine. The signal amplitude was first normalized to baseline and then as a percentage of the maximal response to acetylcholine.

List of PubChem bioassay identifiers generated for this screening project (AID 625, AID 643, AID 1921, AID 1923, AID 1928, AID 1929, AID 1930, AID 1932, AID 1938, AID 1939, AID 2616, AID 449765, AID 449767, AID 449769, and AID 449770.

2.2 Probe Chemical Characterization

Synthetic procedure (large scale) and spectral data for ML173 (CID 45142486).



Probe compound ML173 (CID 45142486) was prepared according to the above scheme and provided the following characterization data: LCMS (>98%) *m/z* = 441 [M+H$^+$] (2.03 min retention, 214 nm). ^{1}H NMR (400MHz, DMSO-d_6): δ = 8.92 (d, *J* = 6.8 Hz, 2H), 8.30 (t, *J* = 5.6 Hz, 1H), 8.09 (d, *J* = 6.4 Hz, 2H), 7.32 (m, 1H), 7.16 (m, 2H), 6.85 (s, 1H), 5.75 (s, 2H), 4.46 (d, *J* = 5.6 Hz, 2H), 2.74 (s, 3H). ^{13}C (100MHz, DMSO-d_6): δ = 165.2, 162.1, 157.8, 157.0, 150.8, 148.8, 148.3, 148.1, 146.4, 146.2, 141.4, 129.3 (d, *J* = 11.0 Hz), 124.5, 124.3, 120.9, 115.6 (d, *J* = 17.0 Hz), 109.5, 65.3, 35.7, 19.9. HRMS calculated for $C_{22}H_{19}N_4O_2F_2S$ [M + H] 441.1197, found 441.1196.

Solubility. Solubility in PBS (at pH = 7.4) was determined to be less than 0.03 µM. However, given the engineered presence of a pyridine nitrogen it was not surprising to find improved solubility under alternate conditions; as its HCl salt, ML173 shows good solubility in these acceptable vehicles (>10 mg/mL in 20% β-cyclodextrin, PEG400/H_2O or pH 3 saline) and >100 µM in DMSO.

Stability. Stability (at room temperature = 23 °C) for ML173 in PBS (no antioxidants or other protectorants and DMSO concentration below 0.1%) is shown in the table below. After 48 hours, the percent of parent compound remaining was 117%, indicating that this probe molecule was stable to the assay conditions, but that assay variability over the course of the experiment ranged from a low of 85% (at 2 hours) to a high of 117% (at 48 hours).

	Percent Remaining (%)						
Compound	**0 Min**	**15 Min**	**30 Min**	**1 Hour**	**2 Hour**	**24 Hour**	**48 Hour**
ML173	100	102	101	95	85	90	117

Reactivity. As assessed through a glutathione (GSH) trapping experiment in phosphate buffered saline (with a substrate concentration of typically 5-50 µM and a GSH concentration of 5 mM, at t = 60 minutes) ML173 was found to not form any detectable GSH adducts.[22]

Compounds added to the SMR collection (MLS#s): 002919691 (ML173, CID 45142486, 500 mg), 002919689, 002919690, 002919692, 002919693, 002919694.

2.3 Probe Preparation

3-amino-*N*-(2,3-difluorobenzyl)-4-methyl-6-oxo-6,7-dihydrothieno[2,3-*b*]pyridine-2-carboxamide (3): To a flask containing 2-mercapto-4-methyl-6-oxo-1,6-dihydropyridine-3-carbonitrile **1** (6.0 mmol) was added DMF (30 mL) followed by TEA (6.6 mmol), and then the mixture was cooled to 0 °C with an ice bath. A solution of 2-chloro-*N*-(2,3-difluorobenzyl)acetamide **2** (6.0 mmol) in DMF (30 mL) was slowly added to the reaction via addition funnel over 30 minutes and then allowed to return to ambient temperature overnight. The reaction was concentrated then partitioned between DCM (100 mL) and H_2O (100 mL), separated, and the aqueous layer washed twice with DCM (100 mL). The organic layer was dried with solid $MgSO_4$, filtered, and concentrated to a tan solid which was washed with EtOAc (3x 30 mL) to afford pure 3-amino-*N*-(2,3-difluorobenzyl)-4-methyl-6-oxo-6,7-dihydrothieno[2,3-*b*]pyridine-2-carboxamide **3** as a light brown solid (50%).

3-amino-*N*-(2,3-difluorobenzyl)-4-methyl-6-(pyridin-4-ylmethoxy)thieno[2,3-*b*]pyridine-2-carboxamide dihydrochloride (ML173, CID 45142486): To a 20 mL microwave vial containing 3-amino-*N*-(2,3-difluorobenzyl)-4-methyl-6-oxo-6,7-dihydrothieno[2,3-*b*]-pyridine-2-carboxamide **3** (2.3 mmol), potassium iodide (2.3 mmol), and cesium carbonate (4.6 mmol) was added DMF (10 mL). To this was added 4-(chloromethyl)pyridine hydrochloride **4** (2.3 mmol)

and the reaction was stirred until bubbling ceased. The vessel was capped and the reaction mixture was heated to 160 °C for 30 min. After cooling to ambient temperature, the reaction was concentrated, and then partitioned between DCM (100 mL) and H_2O (100 mL), separated, and the aqueous layer washed twice with DCM (100 mL). The organic layer was dried with solid $MgSO_4$, filtered, and concentrated to a dark brown solid. This material was crystallized from a mixture of MeOH:DCM:Hexanes with 4N HCl:Dioxanes (5 mL) to afford solid ML173 (CID 45142486) as a light brown solid (60%).

3 Results

Center Summary of Screen: This screen was performed in the pilot phase, the MLSCN, when the MLSMR compound collection at Vanderbilt only contained 12,364 compounds. From the primary M_4 screen of 12,364 compounds, 69 putative M_4 activators were identified with an average Z' score of 0.67±0.093. The confirmation screen (singles at 10 µM) produced 25 active compounds. After a selectivity screen versus M_1, only 4 compounds (**Figure 1**) appeared to be M_4 selective. Chemistry was pursued around these four screening hits **5**-8, and 48 analogs were synthesized (25 analogs of **5**-7 and 23 analogs of **8**); unfortunately, none of these confirmed as selective M_4 agonist or PAMs.

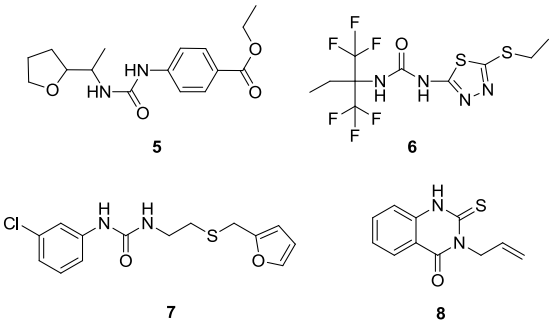


Figure 1. M_4 HTS hits

The project was shelved until a preliminary report from Eli Lilly (personal communication leading to ML108) showed that a new compound, LY2033298, was a robust, but weak, M_4 PAM that is highly selective for *human* M_4 (**Figure 2**). However, to study selective M_4 activation in a preclinical, academic setting, we, and the biomedical community at large, require a tool which is potent and selective for *rat* M_4. Therefore, we initiated a cheminfor-matics and database mining effort based on the LY2033298 scaffold, to attempt to deliver a useful M_4 PAM probe for the MLPCN Network that would possess appropriate potency (rM_4 EC_{50} < 500 nM), and be free from patent restrictions.[16] A substructure search for commercially available compounds in the ChemBridge Corporation's chemical database containing a core similar to LY2033298 delivered a novel lead (CID 714286, not shown) and related analogs, which were free of intellectual property barriers. CID 714286 was a potent PAM of rat M_4 (EC_{50} = 400 nM) which induced a 47-fold leftward shift in the ACh Concentration Response Curve (CRC).

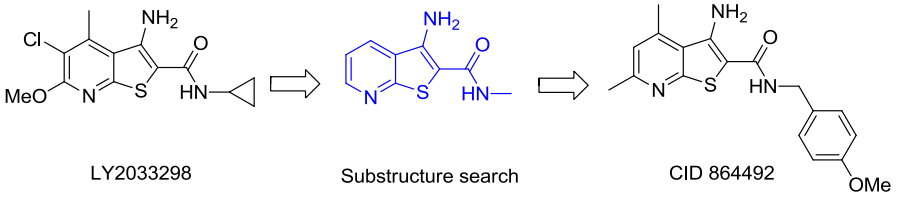
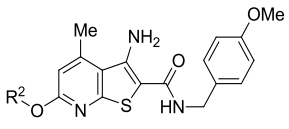
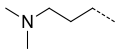
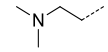
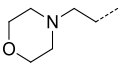
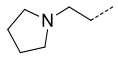
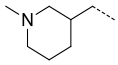
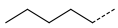
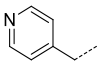
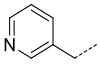
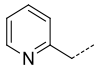


Figure 2. LY2033298 and cheminformatics and database mining approach which lead to the first M_4 probe compound (CID 864492).

Moreover, CID 714286 was highly selective for rat M_4 (EC_{50} > 50 µM for rM_1, hM_2, hM_3 and hM_5), binds at an allosteric site on the M_4 receptor, increases affinity for ACh and increases coupling to G proteins.[17] However, CID 714286 possessed a high log P (4.6) and was virtually insoluble in anything except DMSO.[17] Subsequent optimization of this lead (CID 714286) to our first highly selective rM_4 Probe Molecule (CID 864492, rM_4 EC_{50} = 380 nM, **Figure 2**) was the subject of a previous probe report and has been published elsewhere.[18] Although an excellent probe molecule, compound CID 864492 did display some less than optimal characteristics that we sought to address. Paramount among these was the poor metabolic stability of CID 864492, such that after 90 minutes in the presence of human or rat liver microsomes (*in vitro*) less than 10% of the parent remained. This instability likely resulted in the suboptimal rodent PK for CID 864492, which were adequate but not ideal for in vivo studies.[18]

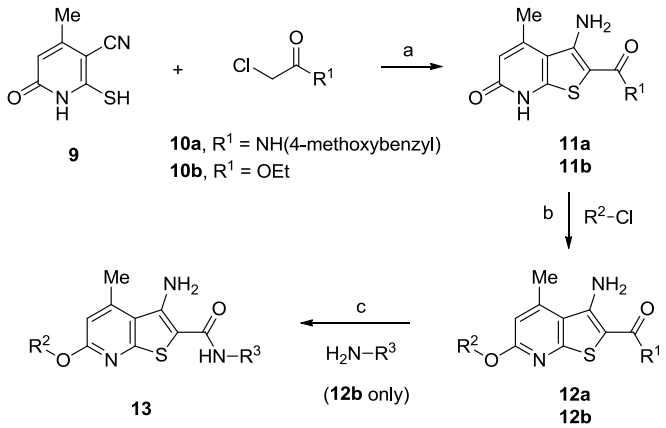
Table 1. Structure and SAR for Analogues **12a.**



CID	R^2	Rat M_4 EC_{50} (µM)[a]	Rat M_4 ACh fold-shift[b]
45142469		> 10	1x
45142470		> 10	ND
45142471		6.5	37x
45142472		8.2	25x
45142473		> 10	ND
45142474		> 10	ND
45142475		> 10	ND
45142476		> 10	ND
44626302		2.0	50x
44626303		2.2	9x
44626304		1.8	5x

[a] Data represent the mean vales from at least 3 experiments with similar results.
[b] Leftward shift of an ACh CRC in the presence of 30 µM compound relative to ACh CRC control.

Scheme 1. Synthesis of Analogues **8a** and **9**.



a) TEA, DMF, 0 °C to rt, 24 h; b) R^2Cl, KI, Cs_2CO_3, DMF, µw 160 °C, 30 min;
c) i. 1 M NaOH/EtOH (3:1), µw 120 °C, 30 min; ii. R^3NH_2, DIC, HOBt, DMF/DIEA (9:1).

Probe Chemical Lead Optimization Strategy: Numerous modifications to the thieno [2,3-*b*]pyridine scaffold, including substitution of the primary amine, deletion or extension at the 4-methyl position and variation of the pyridine to a pyrazine or benzene ring, were all previously found to compromise activity regardless of amide side chain substituent.[18] Metabolite identification experiments indicated that hydroxylation of the 6-methyl group on the pyridine ring was the major oxidative metabolite. Thus, the replacement of this 6-methyl group with ether analogs at this position, to remove the possibility of benzylic oxidation, was explored following the general route of **Scheme 1**. Having tentatively designated the *p*-methoxy-benamide moiety of CID 864492 as a favored substituent based

on its *in vitro* functional activity at rat M_4, we first held this side chain constant (**10a**, R^1 = *p*-methoxybenzylamino) while exploring alternate ethers (R^2, **12a**). Accordingly, the pyridone core **9**[19] was cyclized with α-chloro-*p*-methoxybenzamide (**10a**) to give the key intermediate **11a**. Alkylation of **11a** with various alkyl chlorides, followed by mass-directed preparative HPLC purification, gave the analogs **12a** appearing in **Table 1**.

About half of these analogs possessed EC_{50} values over 10 µM with potentiation effects emerging only at the 10 and 30 µM concentrations from the full CRCs, however encouraging values were seen for some promising, novel structures. Particularly, picolyl analogues CID 44626302, 44626303, and 44626304 each exhibited an EC_{50} value of ~2 µM. A full CRC for CID 44626302 in the presence of a fixed ACh EC_{20} is presented in **Figure 3a**. CID 44626302 elicited a robust potentiation of M_4 activation, elevating the submaximal ACh response to over 130% of the maximum response induced by a high concentration of ACh alone (**Figure 3b**). Looking ahead to *in vivo* studies, the structure of CID 44626302 was particularly attractive as the presence of a basic amine would allow for an HCl salt to confer greater aqueous solubility for vehicle formulation. Based on these potency data, the six compounds that exhibited EC_{50} values below 10 µM were examined for their ability to shift a full ACh CRC to the left when applied at a fixed 30 µM concentration in a similar functional Ca^{2+} assay with rM_4/G_{qi5}-expressing cells (i.e. fold-shift assay). In the case of other allosteric potentiators of GPCRs, compound potency often fails to correlate tightly with fold-shift magnitude. For example, a potentiator with high potency but low efficacy can exhibit next to no fold-shift effect, and conversely one with low potency but high efficacy can induce a substantial fold-shift. Hence, evaluation of fold-shift for novel potentiators having upper single-digit micromolar potencies can sometimes uncover helpful SAR that would have otherwise been missed.

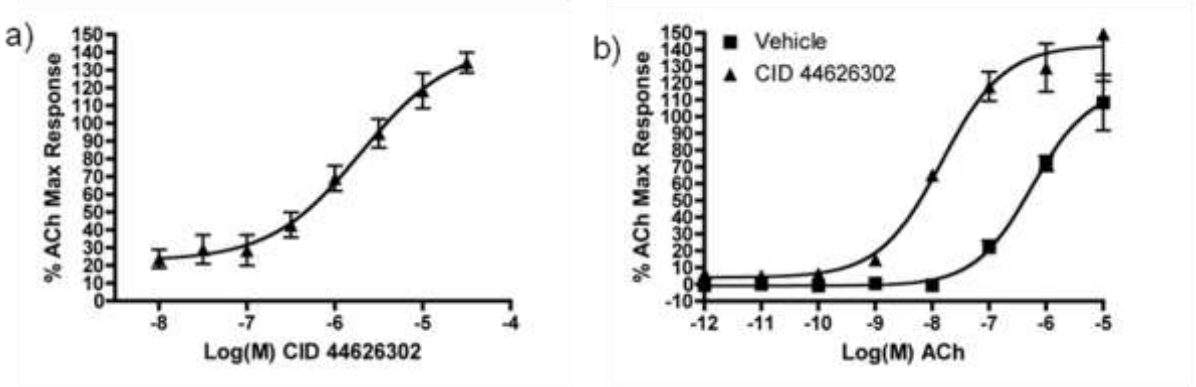
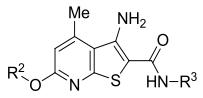
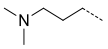
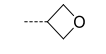
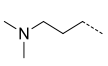
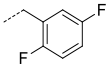
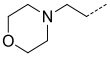
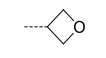
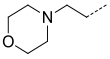
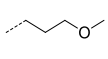
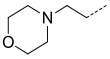
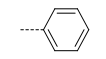
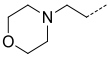
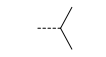
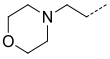
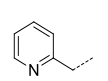
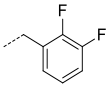
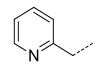
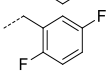
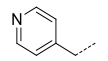
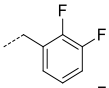
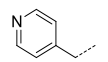
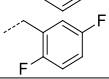


Figure 3. Calcium mobilization assays in rat M_4/G_{qi5}-expressing CHO cells. a) Concentration response curve for CID 44626302 in the presence of a fixed submaximal (~EC_{20}) concentration of ACh (PAM EC_{50} = 2.0 µM). b) Concentration response curve for ACh in the presence or absence of 30 µM CID 44826302 (foldshift = 50x). Data represent means +/- SEM from at least three experiments.

As shown in **Table 1**, purely alkyl ethers (CID 45142474, 45142475 and 45142477) failed to produce measurable potentiation. The same lack of effect was seen with the tertiary amine analogues CID 45142469, 45142470 and 45142473. However, the ether linked morpholino CID 45142471 and pyrrolidine CID 45142472 analogues demonstrated strong leftward fold-shifts (37x and 25x, respectively). Interestingly, movement of the nitrogen from the 2-position or 3-position of the picolyl ethers CID 44626304 and CID 44626303 toward the 4-position of CID 44626302 progressively increased the fold-shift from 5x to 9x and ultimately 50x (**Figure 3b** showing CID 44626302 only), respectively. However, despite retention of robust potentiation properties in terms of fold-shift for analogues CID 45142471, CID 45142472 and particularly CID 44626302, the potency of these analogues was moderately diminished relative to the parent compound CID 864492 (EC_{50} = 380 nM). Furthermore, the SAR for this library underlines the aforementioned importance of considering both fold-shift and potency when evaluating allosteric potentiators. Although each of the three picolyl ether analogues had ~2 µM EC_{50} values, their leftward-shift effects on the ACh CRC revealed dramatic differences in potentiation efficacy. For the next library iteration, we postulated that with the picolyl or ethyl morpholine ether moieties on the left-hand side of the molecule, the p-methoxybenzyl of the right-hand side might no longer be favored for M_4 potency. Therefore, we opted to rescan the amide with select R^3 groups (**Scheme 1**, compound **13**), while sampling each of the three picolyl ether modifications, the morpholino ether, and the dimethylpropylamine ether. The morpholino and 4-picolyl were clear choices based on their degree of fold-shift, but the 2-picolyl and 3-picolyl were also included to be comprehensive. The dimethylpropylamine ether was chosen to provide for the possibility that a different amide side chain may rescue the activity of CID 45142469 (i.e. a matrix-like approach to broaden SAR).

Table 2. Structure and SAR for Analogues **13.**



CID	R^2	R^3	Rat M_4 EC_{50} (µM)[a]	Rat M_4 ACh fold-shift[b]
45142477			2.58	7x
45142478			2.75	14x
45142479			7.42	40x
45142480			6.25	67x
45142489			5.81	51x
45142482			7.15	36x
45142483			8.73	62x
45142484			5.40	28x
45142485			> 10	41x
45142486			2.44	44x
45142487			3.78	64x

[a] Data represent the mean vales from at least 3 experiments with similar results.
[b] Leftward shift of an ACh CRC in the presence of 30 µM compound relative to ACh CRC control.

This second generation library began with the cyclization between pyridone **9** and ethyl chloroacetate (**10b, Scheme 1**) to produce thienopyridone ethyl ester **11b**. To obtain the alkyl ethers **12b**, compound **11b** was alkylated with the five selected side chains from our previously mentioned library (R^2). These scaffolds were saponified and immediately coupled with amines to produce analogs **13** (**Table 2**).

All these compounds possessed an EC_{50} value below 10 μM except for derivative CID 45142485, the difluorobenzyl-substituted 2-picolyl analogue. Similar to earlier libraries, the fold-shift magnitude did not track closely with potency, as shown, for example, with CID 45142483. This *tert*-butyl-substituted morpholine analogue had near 9 μM potency but caused a robust 62-fold shift of the ACh CRC. Furthermore, the two dimethylpropyl analogues CID 45142477 and 45142478 displayed approximately 3 μM potency yet produced only a moderate ACh fold-shift. Interestingly, this dimethylpropylamine moiety at R^2 conferred poor potency (>10 μM) in its parent compound CID 45142469 that possessed the p-methoxybenzyl amide, but this second library discovered side chains at R^3 that rescued activity for this left-hand side modification. In general, difluorinated benzylic substitutions at R^3 were favored, providing analogues with EC_{50} values in the 2–5 μM range at rat M_4 and broad fold-shift values. The 4-picolyl moieties of R^2 with the 2,3-difluoro and 2,5-difluoro substitutions at R^3 of compounds CID 45142486 and CID 45142487 proved most desired when seeking a balance of both potency and potentiation efficacy, consistent with previous SAR. However, the morpholines at R^2 (CIDs 45142479, 45142480, 45142489, 45142482, and 45142483) with bare alkyl and mono-oxygenated side chains at R^3 possessed strong fold-shift effects despite moderately weaker potency compared to CID 45142486 and CID 45142487. Figure 4 presents the CRC for elevation of an ACh ~EC_{20} and fold-shift on a full ACh CRC for analogue CID 45142486. Interestingly, this 2,3-difluorobenzyl substituted analogue did not elevate the maximal response of ACh at the top of the CRC (**Figure 4b**), which contrasts with 4-methoxybenzyl analogue CID 44626302 (**Figure 3b**). Despite generation of a multidimensional library of analogues varying both sides of the lead scaffold, the approximately 400 nm potency (rat M_4) of the first generation compound CID 864492 could not be maintained despite retention of strong potentiation activity in terms of ACh CRC fold-shift (e.g. >50x). Indeed, compounds CID 44626302, 45142480 and 45142487 each caused substantial leftward shift of ACh CRCs when applied at 30 μM, but were approximately an order of magnitude less potent than the first generation compounds at the rat M_4 receptor. These SAR suggest the presence of a possible ~2 μM potency floor for this chemotype with 6-position ether modifications, as variation of the amide side chain failed to provide congers with EC_{50} values below this level.

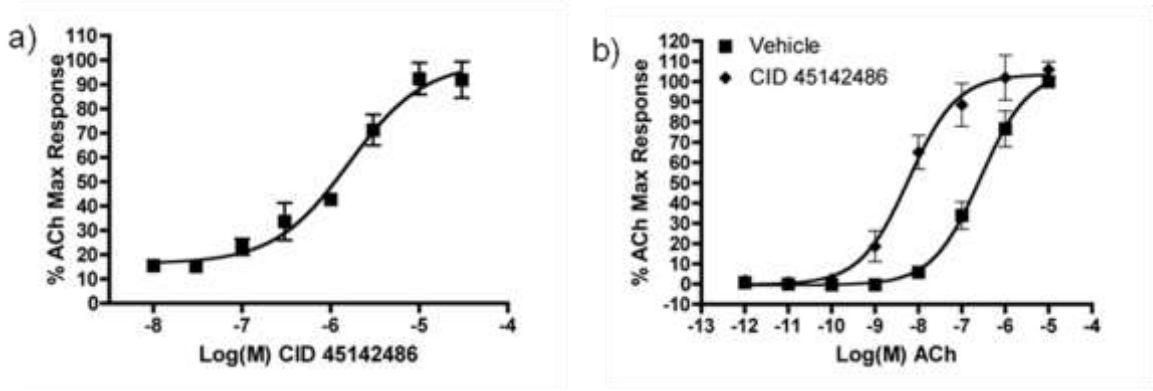


Figure 4. Calcium mobilization assays in rat M_4/G_{qi5}-expressing CHO cells. a) Concentration response curve for CID 45142486 in the presence of a fixed submaximal (~EC_{20}) concentration of ACh (PAM EC_{50} = 2.4 μM). b) Concentration response curve for ACh in the presence or absence of 30 μM CID 45142486 (foldshift = 44x). Data represent means +/- SEM from at least three experiments.

In parallel, we evaluated the microsomal stability of CIDs 44626302, 45142483, 45142486 and 45142487 in both rat and human microsomes. Replacement of the metabolically labile 6-methyl group with the ether linkage did indeed improve metabolic stability for all four analogues CIDs 44626302, 45142483, 45142486 (ML173) and 45142487 (>90% parent remaining after 90 min) as compared to CID 864492 (<10% parent remaining after 90 min). Moreover, incorporation of the basic amine moieties in CIDs 44626302, 45142483, 45142486 and 45142487 also improved solubility providing either homogeneous solutions or uniform microsuspensions, as the HCl salts at 10 mg/mL, across a panel of pharmaceutically acceptable vehicles (β-cyclodextrin, PEG400/H_2O, etc.) relative to CID 864492, which was only soluble in 10% Tween80. In fact, CID 45142486 (ML173) afforded a homogeneous solution at 15 mg/mL in pH 3 saline.

Despite micromolar potency at rat M_4, we evaluated CIDs 44626302, 45142486 (ML173) and 45142487 in our standard reversal of amphetamine-induced hyperlocomotion *in vivo* model, since a long-standing question in the PAM field has centered on whether EC_{50} or fold-shift is more relevant to provide *in vivo* efficacy.[16] As reported previously, CID 864492 (rM_4 EC_{50} = 390 nM, fold-shift of 70x) were efficacious in this model. Interestingly, both CID 44626302 and CID 45142487 produced modest decreases in amphetamine-induced hyper locomotion while CID 45142486 (ML173) had no effect over the time course tested (**Figure 5**). These findings suggest that the diminished potency of these new compounds may have translated to reduced *in vivo* efficacy relative to CID 864492.

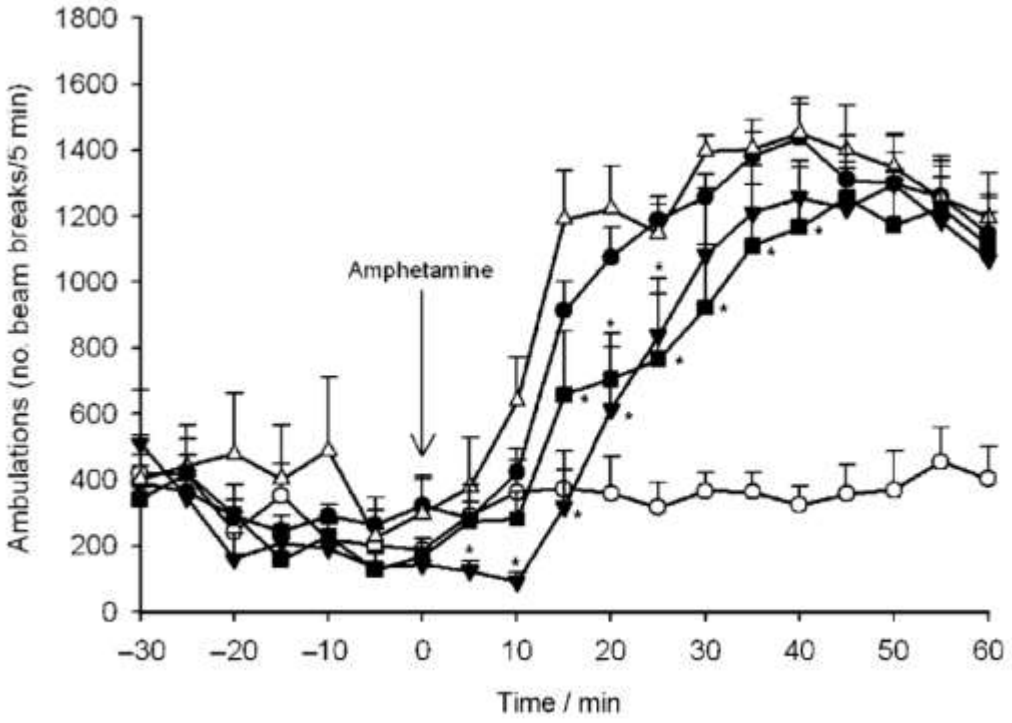


Figure 5. Reversal of amphetamine-induced hyper locomotion rat behavioral study. Pretreatment with vehicle (10% Tween 80 i.p., *n* = 9, dark circle) or a 56.6 mg/kg dose of either CID 44626302 (dark triangle), CID 45142486 (light triangle), or CID 45142487 (dark square) 30 min prior to a 1 mg/kg dose of amphetamine (at *t* = 0, open circle represents control that did not receive amphetamine). Locomotor activity was modestly reversed by CID 44626302 and CID 45142487 (*P*<0.05 versus vehicle plus amphetamine control, Dunnett's test).

Primarily, our efforts reported here were aimed at exploring SAR at rat M_4 and optimizing this series for beneficial DMPK and vehicle formulation properties for *in vivo* rodent behavioral studies. While stability and physiochemical properties were improved, potency at rat M_4 was diminished to a point where *in vivo* efficacy was reduced and, in the case of CID 45142486 (ML173), *in vivo* efficacy was lost. However, rat and human mAChRs do diverge and species differences have been noted for other mAChR PAMs. Therefore, we opted to evaluate representative compounds CIDs 44626302, 45142483, 45142486 (ML173) and 45142487 in analogous functional cell-based Ca^{2+} assays using cells expressing the human M_4 receptor (and promiscuous G_{q15} for Ca^{2+} mobilization readout). To this end, these four compounds were submitted to Millipore Corp. (St. Charles, USA) and assayed by their GPCR Profiler Service, which provided potency and ACh CRC fold-shift values with the human M_4 receptor. Remarkably, each compound possessed EC_{50} values approximately in the 100–200 nM range at human M_4 (**Figure 6a**), more than an order of magnitude greater potency than at the rat M_4 receptor. Each compound also elicited large leftward shifts of the control ACh CRC in human M_4 cells (**Figure 6b**) similar to their respective fold-shifts at rat M_4. In contrast, the first M_4 PAM Probe CID 864492 and about 20 other first generation analogues, displayed near equivalent EC_{50} values at rat and human M_4, suggesting the basic residues in these newer analogues

contact divergent residues in human M_4. While receptor expression levels in the two cell lines is not known, ACh EC_{50} values in the two cell lines are equivalent (rat M_4 ACh, EC_{50} = 154 nM; human M_4 ACh, EC_{50} = 100 nM), and all first generation analogues lacking basic residues were also equipotent. These human M_4 data exemplify the differences that may exist between species in terms of compound potency, efficacy, and other pharmacological parameters, despite relatively high structural similarity between rat and human mAChRs.

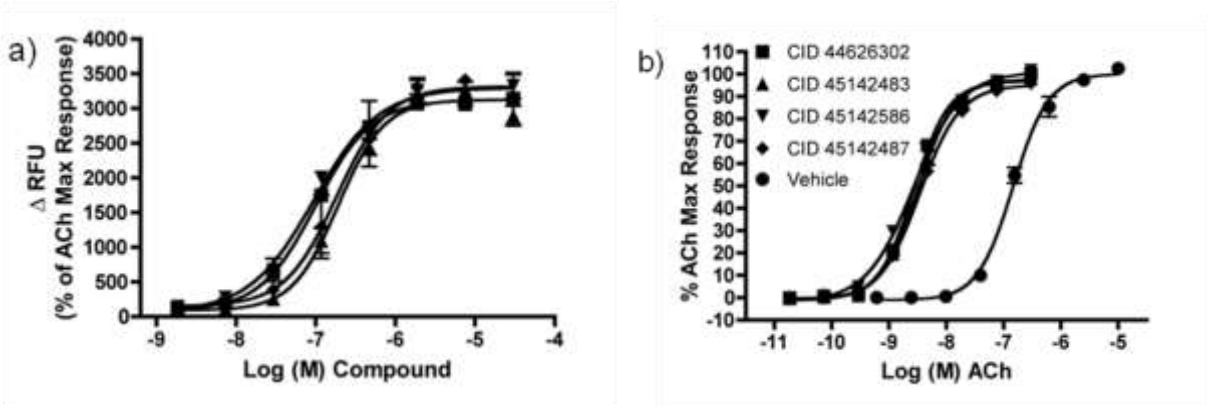


Figure 6. Calcium mobilization assays in human M_4/G_{q15}-expressing CHO cells. a) Concentration response curve for CID 44626302 (square, EC_{50} = 100 nM), CID 45142483 (up triangle, EC_{50} = 192 nM), CID 45142486 (ML173) (down triangle, EC_{50} = 95 nM), and CID 45142487 (diamond, EC_{50} = 183 nM) in the presence of a fixed submaximal (~EC_{20}) concentration of ACh. RFU = relative fluorescent units normalized to baseline. b) Concentration response curve for ACh in the presence or absence of 30 μM CID 44626302 (foldshift = 63x), CID 45142483 (foldshift = 44x), CID 45142486 (foldshift = 60x), and CID 45142487 (foldshift = 49x). Data represent means +/- SEM from at least three experiments.

In addition, CIDs 44626302, 45142483, 45142486 and 45142487 and related second generation analogues remained highly M_4 selective at both human and rat mAChR cell lines (**Figure 7**). Whereas a 30 μM concentration of CIDs 44626302, 45142483, 45142486 (ML173) and 45142487 afforded large leftward shifts (44–63x) of the ACh CRCs of M_4, these same concentrations of compound had no effect on the ACh CRCs of M_1, M_2, M_3 or M_5 (data shown is for rat mAChRs).

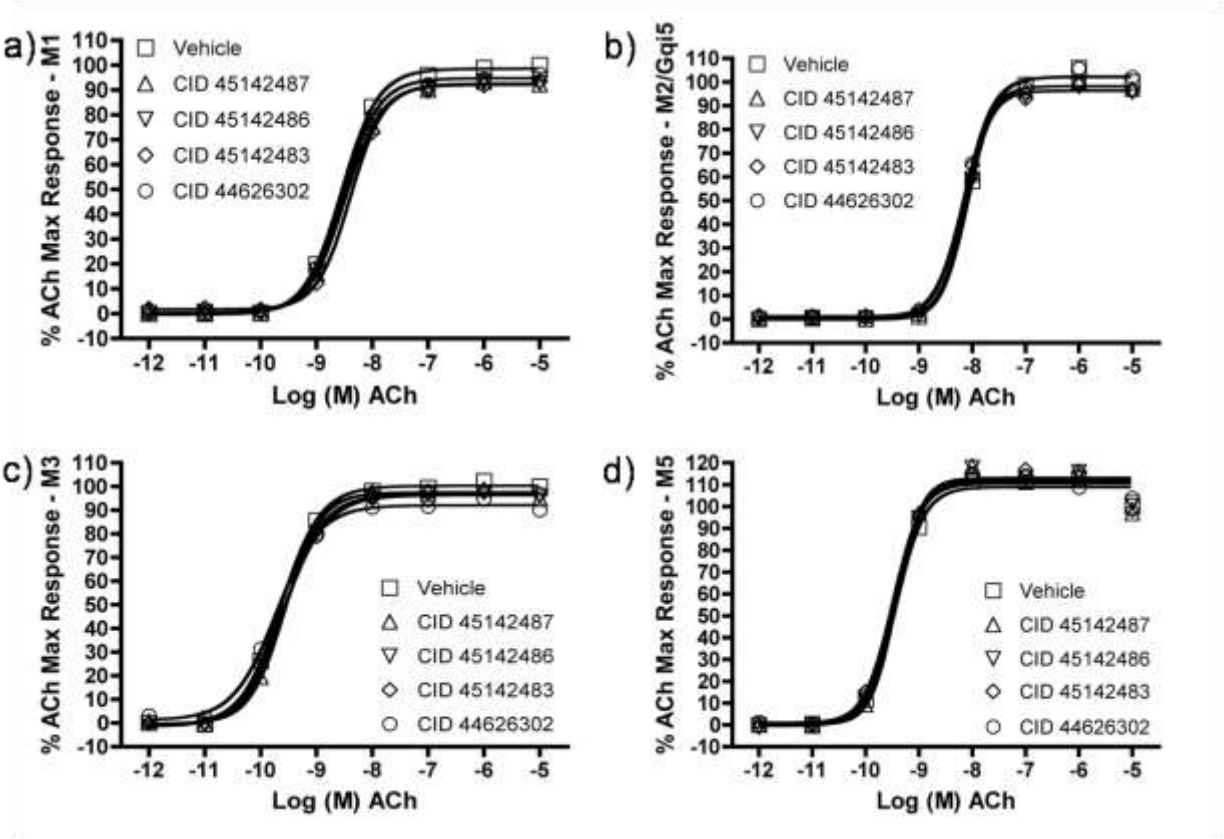


Figure 7. Calcium mobilization assays in a) M_1, b) M_2/Gqi5, c) M_3, and M_5 expressing CHO cells. Concentration response curves for ACh in the absence (square) or presence of 30 μM CID 44626302, CID 45142483, CID 45142486, or CID 45142487, which display no PAM activity at any off-target mAChR subtype (foldshift = 0x for all four compounds). Data represent means +/- SEM from at least three experiments.

The calculated physical properties appearing in Table 3 for the initial M_4 probe molecule (CID 864492) and this current probe (CID 45142486, ML173) were generated using TRIPOS software. Also included in Table 3 are the averages from the MDDR database of compounds both entering Phase I and launched drugs. These numbers indicate that both probes are within the average values for Phase I compounds, except when predicting the number of hydrogen bond donors and calculated solubilities. However, for these two calculated values there are extenuating circumstances that warrant further comment. In the case of hydrogen bond donors, the amide carbonyl may be internally masking one of the hydrogen bond donors on the primary amine through an intramolecular hydrogen bond. Fewer hydrogen bond donors tends to increase the probability of CNS exposure and it has already been shown that the initial probe (CID 864492) and close analogs of the current probe were efficacious in reversing amphetamine-induced hyperlocomotor activity in rats (**Figure 5**), implying some level of CNS exposure. Next, with respect to LogS, although the calculations predict CID 864492 to be more soluble than the current probe CID 45142486, we know from experience that this is not the case, when comparing hydrochloride salts. The pyridine in CID 45142486 (ML173) was

deliberately, and moreover successfully, introduced to improve solubility and points to the importance of not solely relying on neutral forms to judge/predict solubility.

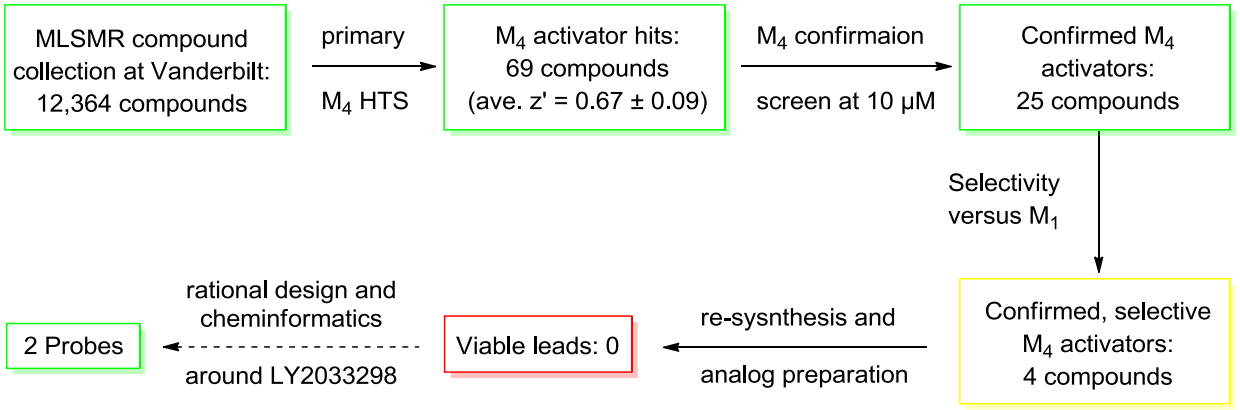
To more fully characterize this novel M_4 PAM probe molecule, CID 45142486 was tested at Ricerca's (formerly MDS Pharma's) Lead Profiling Screen (binding assay panel of 68 GPCRs, ion channels and transporters screened at 10 µM), and was found to not significantly interact with 64 out of the 68 assays conducted (no inhibition of radio ligand binding > 50% at 10 µM).[20] CID 45142486 (ML173) did have activity at the following 4 targets (human targets at 10 µM): Adenosine A_3 (65%), NET (55%), DAT (59%), and Cysteinyl $CysLT_1$ (70%). However it should be pointed out that these are only single-point values and that functional selectivity may be significantly better than suggested by these "% activities." Thus, CID 45142486 (ML173) is highly selective and can be used to dissect the role of hM_4 *in vitro* and potentially *in vivo* with non-human primates.

Table 3: Calculated Property Comparison with MDDR Compounds

Property	CID 864492	CID 45142486 (ML173)	MDDR Phase I	MDDR Launched
MW	341.4	440.5	438.98	415.20
cLogP	2.68	3.12	3.21	2.21
TPSA	77.2	90.1	97.06	91.78
Hdon	3	3	2.12	2.13
Hacc	5	6	7.06	6.47
LogS	-5.02	-6.11	-4.96	-3.73
NrotB	5	7	7.08	5.71

In summary, a lead optimization campaign around the initial M_4 PAM probe CID 864492 provided novel analogues with improved metabolic stability and physiochemical properties, but diminished efficacy at rat M_4 (EC_{50} values ~2 µM) while retaining comparable fold-shift (14 – 67x) of the ACh CRC. Moreover, though weak at rat M_4, several close analogues displayed modest *in vivo* efficacy in reversing amphetamine-induced hyper locomotion, a classic preclinical antipsychotic model. Surprisingly, we noted significant species differences within this new series of M_4 PAMs, where analogues such as CID 45142486 (ML173) displayed an order of magnitude greater potency at human M_4 (EC_{50} = 95 nM) than at the rat M_4 receptor (EC_{50} = 2.4 µM) with comparable fold-shifts (human, 60x; rat, 44x) and high M_4 mAChR subtype selectivity.

3.1 Summary of Screening Results



3.2 Dose Response Curves for Probe

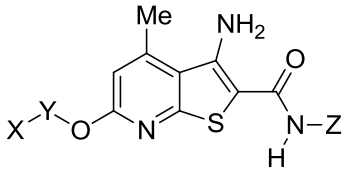
See **Figures 4**, **6** and **7** (*vide supra*).

3.3 Scaffold/Moiety Chemical Liabilities

No chemical liabilities for the probe molecule have been identified at the present time.

3.4 SAR Table

Table 4: SAR Analysis for rat M_4 Positive Allosteric Modulators.



Entry	CID	SID	VU Number	*	X	Y	Z	Rat M4 EC50 (µM) **	Rat M4 ACh fold-shift ***
1	45142469	92392513	VU0152117	S	dimethylamino	n-propyl	4-methyoxybenzyl	> 10	1x
2	45142470	92392514	VU0152118	S	dimethylamino	ethyl	4-methyoxybenzyl	> 10	-
3	45142471	92392515	VU0152119	S	morpholino	ethyl	4-methyoxybenzyl	6.5	37x
4	45142472	92392516	VU0152120	S	pyrrolidin-1-yl	ethyl	4-methyoxybenzyl	8.2	25x
5	45142473	92392517	VU0152121	S	1-methylpiperidin-3-yl	methylene	4-methyoxybenzyl	> 10	-
6	45142474	92392518	VU0152122	S	-	n-propyl	4-methyoxybenzyl	>10	-
7	45142475	92392519	VU0152123	S	-	i-propyl	4-methyoxybenzyl	>10	-

8	45142476	92392523	VU0152133	S	-	n-pentyl	4-methyoxybenzyl	>10	-
9	44626302	92392520	VU0152129	S	pyridin-4-yl	methylene	4-methyoxybenzyl	2.0	50x
10	44626303	92392521	VU0152130	S	pyridin-3-yl	methylene	4-methyoxybenzyl	2.2	9x
11	44626304	92392522	VU0152131	S	pyridin-2-yl	methylene	4-methyoxybenzyl	1.8	5x
12	45142477	92392524	VU0359439	S	dimethylamino	n-propyl	oxetan-3-yl	2.58	7x
13	45142478	92392525	VU0359451	S	dimethylamino	n-propyl	2,5-difluorobenzyl	2.75	14x
14	45142479	92392526	VU0359453	S	morpholino	ethyl	oxetan-3-yl	7.42	40x
15	45142480	92392527	VU0359454	S	morpholino	ethyl	3-methoxypropyl	6.25	67x
16	45142489	92392537	VU0359455	S	morpholino	ethyl	phenyl	5.81	51x
17	45142482	92392529	VU0359459	S	morpholino	ethyl	i-propyl	7.15	36x
18	45142483	92392530	VU0359460	S	morpholino	ethyl	t-butyl	8.73	62x
19	45142484	92392531	VU0359479	S	pyridin-2-yl	methylene	2,3-difluorobenzyl	5.40	28x
20	45142485	92392532	VU0359480	S	pyridin-2-yl	methylene	2,5-difluorobenzyl	> 10	41x
21	45142486	92392533	VU0359508	S	pyridin-4-yl	methylene	2,3-difluorobenzyl	2.44	44x
22	45142487	92392534	VU0359509	S	pyridin-4-yl	methylene	2,5-difluorobenzyl	3.78	64x

* S = synthesized, P = purchased. ** Data represent the mean values from at least 3 experiments with similar results [AID 2616]. *** Leftward shift of an ACh CRC in the presence of 30 µM compound relative to ACh CRC control [AID 449769].

3.5 Cellular Activity

This series of positive allosteric modulators displayed functional activity (Ca^{+2} mobilzation) in CHO cells stably expressing the human and rat M_4 receptors [AID 2616 and 449769]. Although not demonstrated with the probe molecule (ML173) close analogs did show modest decreases in amphetamine-induced hyper locomotion in rats, implying at least some level of CNS exposure was obtained, while not showing any signs of overt toxicity or general sedation.

3.6 Profiling Assays

The probe molecule (ML173) and 3 close analogs were profiled in functional cell-based Ca^{+2} mobilization assays across the five muscarinic subtypes at Millipore Corp. (St. Charles, USA) and were found to elicit measureable potentiation at only the M_4 receptor subtype. Furthermore, ML173 was tested at Ricerca's (formerly MDS Pharma's) Lead Profiling Screen (binding assay panel of 68 GPCRs, ion channels and transporters screened at 10 µM), and was found to not significantly interact with 64 out of the 68 assays conducted (no inhibition of radio ligand binding > 50% at 10 µM).[20] CID 45142486 (ML173) did have activity at the following 4 targets (human targets at 10 µM): Adenosine A_3 (65%), NET (55%), DAT (59%), and Cysteinyl $CysLT_1$ (70%). However it should be pointed out that these are only single-point values and that functional selectivity may be significantly better than suggested by these "% activities."

4 Discussion

4.1 Comparison to existing art and how the new probe is an improvement

The first selective M_4 PAM disclosed was LY2033298 (see Figure 2). Similar to ML173, this compound showed significantly reduced potency between the rat and human receptors. Although low nanomolar potency was observed at the human M_4 receptor, this decrease in potency at the rodent receptor necessitated the co-administration of a submaximal dose of oxotremorine (a non-selective muscarinic agonist) to observe the *in vivo* M_4 potentiating effects of LY2033298 in a condition avoidance response rodent model.[21] Additionally, when the same experiment was conducted in M_4 Knock Out (KO) mice the actions of LY2033298 were significantly attenuated but not abolished as might be expected for a highly selective M_4 PAM. Still, LY2033298 served as the inspiration, along with cheminformatics and database mining efforts, to produce our first M_4 PAM probe (ML 108). This compound possessed a rM_4 EC_{50} = 380 nM, and more importantly showed efficacy in a different antipsychotic rat model (reversal of amphetamine-induced hyperlocomotor activity) without needing to be co-dosed with a non-selective muscarinc agonist. The major shortcomings associated with ML 108 were metabolic stability and solubility. Relative to our initial M_4 PAM probe (ML 108) this second generation probe (ML173) possesses improved metabolic stability and physiochemical properties, but diminished efficacy at rat M_4 (EC_{50} values ~2 µM) while retaining a comparable fold-shift (44x) of the ACh CRC. Though weakly efficacious at rat M_4, several close analogues displayed modest *in vivo* efficacy in reversing amphetamine-induced hyper locomotion, a classic preclinical antipsychotic model. We noted significant species differences within this new series of M_4 PAMs, where analogues such as CID 45142486 (ML173) displayed an order of magnitude greater potency at human M_4 (EC_{50} = 95 nM, surpassing our initial M_4 probe) than at the rat M_4 receptor (EC_{50} = 2.4 µM) with comparable fold-shifts (human, 60x; rat, 44x) and high M_4 mAChR subtype selectivity. Lastly, this probe (ML173), along with our initial probe (ML 108), is not encumbered with patent restrictions and rests firmly in the public domain.

4.2 Mechanism of Action Studies

It is believed that this probe is functioning by positive allosteric modulation of the muscarinic acetylcholine M_4 receptor given its lack of activity on the other muscarinic receptor subtypes ($M_{1\text{-}3,5}$), its dependence on the presence of ACh to elicit functional activity and its nearly clean profile in the Ricerca lead profiling screen.

4.3 Planned Future Studies

At the present time no further studies are planned with this probe molecule. However, given its excellent selectivity profile it will continue to be used as a reference standard for *in vitro* experiments exploring hM_4 receptor activation and downstream signaling. Ultimately, if the

opportunity arises to study selective M_4 receptor activation in non-human primates this probe will be among our top candidates for initial testing.

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